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[COMMUNICATION]

Superoxide Production by the Haemocytes of the Freshwater Snail, *Biomphalaria glabrata*, Stimulated by Miracidia of *Schistosoma mansoni*

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ABSTRACT—The production of superoxide (O_2^-) by the haemocytes of *Biomphalaria glabrata*, an intermediate host of *Schistosoma mansoni*, was demonstrated by nitroblue tetrazolium (NBT) test. Intact, 4,000 R irradiated and formalin-fixed miracidia (snail-infective larvae) of *S. mansoni* were used as the stimuli. NBT-reduction was observed in the haemocytes incubated with 4,000 R irradiated or formalin-fixed miracidia, whereas only little effect was shown by intact ones. The reduction was inhibited by superoxide dismutase. The results suggest that self/non-self recognition by the haemocytes is involved in the O_2^- production.

INTRODUCTION

It is generally accepted that cellular responses, such as phagocytosis or encapsulation, play a major part in molluscan internal defence. However, little is known about the biochemical processes which occur during the responses [1, 2].

Vertebrate phagocytes stimulated with various agents show a respiratory burst that results in the production of active oxygen metabolites, which contribute to microbicidal activities [3–5]. On the other hand, insect [6] and bivalve [7] haemocytes were reported to be devoid of such antimicrobial system known in vertebrate phagocytes.

Recently, however, Nakamura *et al.* [8] found that haemocytes of the scallop, *Patinopecten yessoensis*, produce and release hydrogen peroxide (H_2O_2) under phagocytic stimulation *in vitro*. Dikkeboom *et al.* [9] also detected the production of superoxide (O_2^-) and H_2O_2 by the stimulated haemocytes of the pond snail, *Lymnaea stagnalis*. These results imply that molluscan haemocytes may have some bactericidal or cytotoxic systems comparable to vertebrate phagocytes.

The first aim of this study was to detect the possible production of O_2^- by stimulated haemocytes of *Biomphalaria glabrata*, an intermediate host snail of the human blood fluke, *Schistosoma mansoni*. If it is really produced by the cells, what is the role of O_2^- production in the defence mechanisms of the snail? To answer the question, we tried to stimulate the haemocytes of *B. glabrata* with miracidia (snail-infective larvae) of *S. mansoni*.

MATERIALS AND METHODS

Snails: The pulmonate snails, *B. glabrata* (Puerto Rican strain), which were provided from Department of Parasitology, Hamamatsu University School of Medicine, and Department of Medical Biology, Institute of Basic Medical Sciences, University of Tsukuba, have been maintained in our laboratory since 1985. They were kept in aquaria at 25°C and fed on an artificial diet for the silkworm [10] *ad libitum*. Their susceptibility to *S. mansoni* infection tested in our labolatory was 86% (95% confidence interval: $80\% < p < 92\%$). Adult specimens (shell diameters: 10–15 mm) were placed in distilled water for more than 12 hr before experiments.

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Haemolymph collection

The shell surface of the snail was swabbed with 70% ethanol and dried before use. The snail retracts deeply into its shell and extrudes haemolymph, when it is touched with the point of a glass capillary [11]. In this way, about 15–25 μl of haemolymph can be obtained from an adult specimen. Haemolymph collected from 20 or more snails was pooled in an ice-cooled, sterile polyethylene sample tube and used within 1 hr. The total haemocyte counts of the haemolymph was *ca.* 1×10^6 per ml.

Stimuli

S. mansoni (Puerto Rican strain) was provided from Department of Parasitology, Institute of Medical Science, University of Tokyo and has been maintained in our laboratory since 1985. Miracidia were obtained from minced livers of ICR mice (8 weeks post infection with cercariae of *S. mansoni*) by immersing in aged tap water. They were washed twice in sterile, distilled water and used as the intact miracidia. For the preparation of irradiated miracidia, the suspension was adjusted to contain approximately 50 miracidia per ml and exposed to 4,000 R X-ray irradiation generated by Hitachi MBR-1505 R (Hitachi Medico Ltd., Japan). The miracidia were fixed by 3% formalin solution. After 60 min of fixation, they were washed three times and resuspended in sterile, distilled water.

Nitroblue tetrazolium test

O_2^- generation was detected by the histochemical assay using nitroblue tetrazolium (NBT). On a glass slide, 20 μl of haemolymph was placed, then 10 μl of sterilized Sminia's snail saline, pH 7.4 (SS; NaCl 249.5 mg, KCl 15.5 mg, $MgCl_2$ 40.7 mg, $CaCl_2$ 64.7 mg in 100 ml of distilled water; pH was adjusted with 0.2 M Tris-HCl, pH 8.0) [12] with or without 2.0 μg of superoxide dismutase (SOD; 3,000 U/mg protein, from bovine erythrocytes; Sigma) and 5 miracidia were added. As a control, miracidia were omitted and SS alone was added to the haemolymph. After 15 min of preincubation, 10 μl of 0.2% NBT (Sigma) dissolved in SS was overlaid and incubated for 60 min. All the incubation was done at 25°C in a moist chamber. The slides were fixed in 100% methanol, mounted in glycerol and examined under a phase-contrast microscope. If O_2^- was produced by the cells, yellow, soluble NBT is reduced to blue-black, insoluble formazan. The cells having intracellular formazan glanule (s) were considered to be positive for O_2^- production (Fig. 1). At least 500 cells were obsereved and the percentage of positive cells was determined. Experiments were done in triplicate. Each value is shown by the mean$\pm$SD.

RESULTS

Both intact and irradiated miracidia transformed into sporocysts and were alive during the incuba-

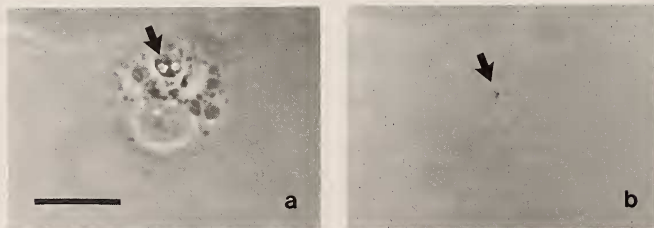

FIG. 1. Phase contrast(a) and light(b) micrographs of a haemocyte of *Biomphalaria glabrata*, which has intracellular formazan granule (arrows) indicating superoxide production. (Bar=10 μm).

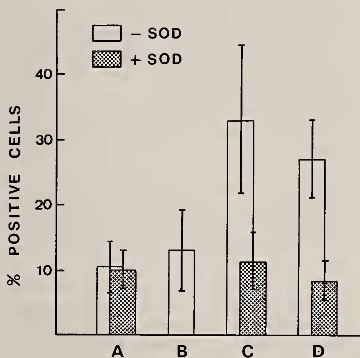

FIG. 2. Percentages of the haemocytes of *Biomphalaria glabrata* having intracellular formazan granule(s). The cells were incubated with (open columns) or without (shaded columns) superoxide dismutase (SOD; final concentration: 50 μg/ml) and stimulated as follows: A, controls without stimuli; B-D, stimulated by intact, 4,000 R irradiated or formalin-fixed miracidia of *Schistosoma mansoni,* respectively. After 15 min of preincubation, nitroblue tetrazolium solution (final concentration: 0.05%) was added and incubated for 60 min at 25°C. Each value represents the mean of triplicate experiments, SD is indicated as a vartical bar.

tion with the haemocytes. Haemocytic encapsulation and adherence against the parasites were never observed in this period.

Percentages of formazan-positive cells are shown in Figure 2. A number of positive cells were observed among the haemocytes incubated with irradiated or formalin-fixed miracidia and the percentages were 33.0±11.4 and 27.0±6.0, respectively. Only a little effect was shown by intact miracidia (13.0±6.3%).

SOD inhibited the NBT-reduction by the haemocytes incubated with irradiated or formalin-fixed miracidia; the percentages of the positive cells decreased to 11.4±4.5 and 8.4±3.2, respectively. The positive rate in the controls (10.5± 4.1%) was unchanged by SOD.

DISCUSSION

In the host-parasite relation between *B. glabrata* and *S. mansoni,* several strains of the host snail are known to be genetically different in their susceptibility to the parasite [13, 14]. When miracidia penetrate to resistant snails, the sporocysts are encapsulated and killed by the haemocytes. The haemocytes of resistant snails were also reported to be cytotoxic to the sporocysts *in vitro* [15]. However, the haemocytes of susceptible ones show little resistance to the parasites *in vivo* [1, 16, 17]. On the other hand, irradiated miracidia of *S. mansoni* are encapsulated by the haemocytes of susceptible snail and fail to develop, though they can penetrate into the host [18]. These cellular responses have been considered to be based on self/non-self recognition by the host, but the killing mechanism(s) remains to be elucidated [2, 15].

The present results clearly indicate that the haemocytes are stimulated by treated (alive and dead) miracidia to produce O_2^-. The NBT-reduction by the haemocytes stimulated with the miracidia is considered to be similar to the reaction observed in the haemocytes of *L. stagnalis* stimulated with various phagocytic particles or phorbol myristate acetate [9].

Miracidia are generally encapsulated by a number of haemocytes, when the host defence reaction is provoked. NBT-reduction is usually seen in the adherent cells and at the haemocyte/parasite interface in vertebrate haemocytic encapsulation against parasite [19, 20]. In our experiments, probably because of the brief incubation period, neither encapsulation nor adhesion of the haemocytes against any miracidia or sporocysts was observed. However, larger number of formazan positive cells were found around the irradiated or formalin-fixed miracidia than in the other part. This suggests that the positive cells, though they did not adhere to the surface of the parasites, might have some chance to encounter the treated parasites and stimulated during the incubation.

In contrast, the haemocytes seem to be rarely stimulated by the intact miracidia, because the cells incubated with intact ones showed only a slight reduction of NBT. The difference in the haemocytic reactions against intact or treated

miracidia might be to non-self recognition by the haemocytes. Since intact miracidia are recognized as 'self' by the haemocytes of susceptible hosts, they do not induce any cellular reactions [16, 17]. In the similar manner, the haemocytes stimulated by the intact miracidia would have failed to produce O_2^-. Whereas both sporocysts transformed from irradiated miracidia and formalinfixed ones are recognized as 'non-self' by the host [1, 18]. Therefore, these treated parasites are considered to have provoked the O_2^- production by the haemocytes.

In conclusion, we detected the O_2^- production by the stimulated haemocytes of *B. glabrata.* The results, moreover, indicate that the induction of the cellular response involves self/non-self recognition by the haemocytes. Although the cytotoxicity or microbicidal activities of the oxygen radicals produced by the snail haemocytes have to be further investigated, one can expect that these metabolites would have some roles in the defence system of gastropods.

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